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Identification of a Gatekeeper Residue That Prevents Dehydrogenases from Acting as Oxidases*[S]◆

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Nicole G. H. Leferink[‡], Marco W. Fraaije[§], Henk-Jan Joosten[¶], Peter J. Schaap[¶], Andrea Mattevi[‖], and Willem J. H. van Berkel[‡1]

From the [‡]Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, the [§]Laboratory of Biochemistry, Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, the [¶]Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands, and the [‖]Department of Genetics and Microbiology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy

The oxygen reactivity of flavoproteins is poorly understood. Here we show that a single Ala to Gly substitution in L-galactono-γ-lactone dehydrogenase (GALDH) turns the enzyme into a catalytically competent oxidase. GALDH is an aldonolactone oxidoreductase with a vanillyl-alcohol oxidase (VAO) fold. We found that nearly all oxidases in the VAO family contain either a Gly or a Pro at a structurally conserved position near the C4a locus of the isoalloxazine moiety of the flavin, whereas dehydrogenases prefer another residue at this position. Mutation of the corresponding residue in GALDH (Ala-113 → Gly) resulted in a striking 400-fold increase in oxygen reactivity, whereas the cytochrome *c* reductase activity is retained. The activity of the A113G variant shows a linear dependence on oxygen concentration (k_{ox} = 3.5 × 10^5 M^{-1} s^{-1}), similar to most other flavoprotein oxidases. The Ala-113 → Gly replacement does not change the reduction potential of the flavin but creates space for molecular oxygen to react with the reduced flavin. In the wild-type enzyme, Ala-113 acts as a gatekeeper, preventing oxygen from accessing the isoalloxazine nucleus. The presence of such an oxygen access gate seems to be a key factor for the prevention of oxidase activity within the VAO family and is absent in members that act as oxidases.

The flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH;[2] EC 1.3.2.3) catalyzes the terminal step in the biosynthesis of vitamin C (L-ascorbate) in plants. Besides producing this essential nutrient, GALDH is required for the accumulation of plant respiratory complex I (1). GALDH is an aldonolactone oxidoreductase that belongs to the vanillyl-alcohol oxidase (VAO) flavoprotein family (2). Members of this family share a two-domain folding topology with a conserved FAD binding domain and a cap domain that defines the substrate specificity (3). VAO family members include enzymes involved in carbohydrate metabolism and lignin degradation and enzymes that participate in the synthesis of antibiotics and alkaloids (4). Most VAO members contain a covalently tethered FAD and act as oxidases that use molecular oxygen to reoxidize the flavin, resulting in the production of hydrogen peroxide. In contrast to related aldonolactone oxidoreductases like L-gulono-γ-lactone oxidase from animals (5) and D-arabinono-γ-lactone oxidase from yeast (6), GALDH reacts poorly with molecular oxygen and contains non-covalently bound FAD (7). No crystal structure is available for the aldonolactone oxidoreductase subfamily, and little is known about the nature of the active site and the catalytic mechanism.

GALDH is localized in the mitochondrial intermembrane space, where it feeds electrons into the respiratory chain. Its subcellular localization could provide a rationale why GALDH is a dehydrogenase and not, like related enzymes, an oxidase. The latter activity would result in high levels of mitochondrial hydrogen peroxide that promote GALDH inactivation (8) and induce aging, senescence, and cell death (9, 10). Furthermore, it has been shown that the exploitation of the electron transport chain of GALDH via cytochrome *c* is essential for the proper functioning of plant mitochondria (11).

GALDH from *Arabidopsis thaliana* has several properties in common with flavoprotein oxidases, including the stabilization of the red anionic flavin semiquinone and the formation of a flavin N5-sulfite adduct (7) but reacts 3–4 orders of magnitude slower with molecular oxygen than what is typically found for oxidases. The reaction of flavoprotein oxidases with molecular oxygen generally follows second order kinetics with rate constants in the range of 10^5 M^{-1} s^{-1} for most oxidases to 10^6 M^{-1} s^{-1} for glucose oxidase (12).

Up to now, there are no clear rules that enable us to predict whether or how a flavoprotein reacts with molecular oxygen. A combination of structural and dynamic features like properly positioned positive charges, solvation of the active site, and protein breathing might play a role (12). The existence of (hydrophobic) oxygen channels from the surface to the active site has been proposed for several flavoenzymes, including the VAO members cholesterol oxidase (CO) (13) and alditol oxidase (AldO) (14).

Structure-inspired attempts to alter the oxidase activity of flavoenzymes have been mainly focused on decreasing the oxygen reactivity. Blocking putative oxygen binding sites or oxygen

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text, a supplemental table, and a supplemental figure.

[1] To whom correspondence should be addressed. Tel.: 31-317-482861; Fax: 31-317-484801; E-mail: willem.vanberkel@wur.nl.

[2] The abbreviations used are: GALDH, L-galactono-γ-lactone dehydrogenase; VAO, vanillyl-alcohol oxidase; CO, cholesterol oxidase; AldO, alditol oxidase.

channels resulted in mutant proteins with poor catalytic properties and which are still active with molecular oxygen (15–17). Replacing a Lys in the active site of monomeric sarcosine oxidase decreased the oxygen reactivity by more than 1000-fold (18). Slight improvements in the oxygen reactivity were reported for D-amino acid oxidase (19), where a directed evolution approach was followed to obtain variants with increased oxygen affinity. A Tyr to Lys mutation increased the oxygen reactivity of medium-chain acyl-CoA dehydrogenase due to a better exposure of the flavin to solvent, but the reaction is far from efficient (20).

In this study, we investigated the molecular determinants for the poor oxygen reactivity of GALDH from *A. thaliana*. Because structural information for the aldonolactone oxidoreductases is lacking, we used a structure-based multiple sequence alignment strategy to find structural correlations with oxidase activity within the VAO family. Strikingly, we found that nearly all oxidase members of the VAO family contain a glycine or proline at a structurally conserved position near the C4a atom of the isoalloxazine ring of the flavin, whereas members that do not react with oxygen contain another residue at this position. The role of the corresponding residue in GALDH, Ala-113, in the reaction with molecular oxygen was addressed by site-directed mutagenesis. We describe for the first time the characterization of a GALDH variant capable of efficiently utilizing oxygen as electron acceptor, rendering a catalytically competent L-galactono-γ-lactone oxidase.

EXPERIMENTAL PROCEDURES

Chemicals—Benzyl viologen, bovine heart cytochrome *c*, FAD, L-galactono-1,4-lactone, and xanthine were purchased from Sigma-Aldrich. Resazurin was obtained from Acros Organics, and xanthine oxidase, catalase and superoxide dismutase were from Roche Applied Science. All other chemicals were from commercial sources and of the purest grade available.

Sequence Comparisons—A structure-based multiple sequence alignment of the FAD domain of the VAO family was generated with the 3DM software (21). The 3DM alignment can be viewed at the 3DM Alignment Program page (FAD-linked oxidases database). Additional Clustal W multiple sequence alignments were performed to find corresponding residues at structurally conserved positions for VAO members lacking in the 3DM alignment.

Cloning and Site-directed Mutagenesis—The cDNA encoding mature GALDH (At3g47930) has been cloned previously into the pET23a(+) vector (Novagen) to yield pET-GALDH-His_6 (7). The GALDH A113G variant was constructed using pET-GALDH-His_6 as template with the QuikChange II method (Stratagene) using the oligonucleotides A113G_fw (forward) (5′-CTC TTC AGA ACT TTG GCT CCA TTA GAG-3′) and A113G_rv (reverse) (5′-CTG CTC TCT AAT GGA GCC AAA GTT CTG-3′). Successful mutagenesis was confirmed by automated sequencing. The resulting construct pET-GALDH_A113G-His_6 was electroporated to *Escherichia coli* BL21(DE3) cells (Novagen) for recombinant expression.

Enzyme Production and Purification—For enzyme production, *E. coli* BL21(DE3) cells, harboring a pET-GALDH plasmid, were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an A_{600} of 0.7 was reached. Expression was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and the incubation was continued for 16 h at 37 °C. The recombinant His_6-tagged proteins were purified essentially as described before (7).

Protein Analysis—SDS-PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli (22). Proteins were stained using Coomassie Brilliant Blue R-250. Total protein concentrations were estimated using the Bradford protein assay from Bio-Rad with bovine serum albumin as standard. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns (Bio-Rad).

Spectral Analysis—Absorption spectra were recorded at 25 °C on a Hewlett Packard 8453 diode array spectrophotometer in 50 mM sodium phosphate buffer, pH 7.4. Spectra were collected and analyzed using the UV-visible ChemStation software package (Hewlett Packard). The molar absorption coefficient for the mutant protein was determined by recording absorption spectra in the presence and absence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} at 450 nm. Enzyme concentrations were routinely determined by measuring the absorbance at 450 nm using a molar absorption coefficient of 12.9 mM^{-1} cm^{-1} for wild-type GALDH (7).

Reduction Potential Determination—The reduction potentials of wild-type GALDH and the A113G variant were determined using the method of Massey (23). Briefly, a 1-ml subasealed cuvette containing 5 μM GALDH, 400 μM xanthine, 2.5 μM benzyl viologen, and 5 μM redox dye in 50 mM sodium phosphate, pH 7.4, was made anaerobic by flushing with oxygen-free nitrogen. To scavenge final traces of oxygen, 1 mM vanillyl-alcohol and catalytic amounts of eugenol oxidase (24) were added to the reaction mixture. To start the reaction, 2 μl of xanthine oxidase (20 units/ml) was added, and spectra were collected every 2 min during the reduction using a PerkinElmer Life Sciences Lambda Bio 40 spectrophotometer. The potentials at 50% reduction of GALDH (E_m) were calculated using the Nernst equation,

$$E_h = E_m + 2.303 \times (R \times T/n \times F) \times \log([ox]/[red]), \quad \text{(Eq. 1)}$$

where E_h is the measured potential, E_m is the midpoint potential of the enzyme, R is the gas constant (8.31 J K^{-1} mol^{-1}), T is the temperature in K, n is the number of electrons to go from the oxidized species to the reduced species, F is the Faraday constant (96.5 kJ V^{-1} mol^{-1}), and [ox] and [red] are the concentrations of oxidized and reduced enzyme, respectively.

Activity Measurements—GALDH activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm (7). Because dithiothreitol interferes with the reaction, it was removed from the enzyme solution by Bio-Gel P-6DG gel filtration immediately prior to use. Initial velocities were calculated using a molar difference absorption coefficient ($\Delta\epsilon_{550}$) of 21 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that

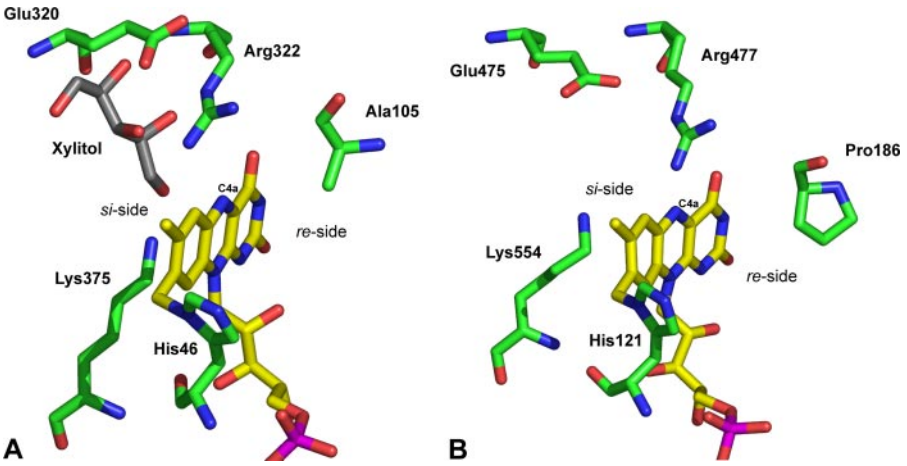

FIGURE 1. **Active sites of GALDH homologs.** *A*, AldO in complex with xylitol (Protein Data Bank (PDB) number 2vfs) (14). *B*, cholesterol oxidase (PDB number 1i19) (13). For clarity, only selected amino acid residues are shown. The C4a atom of the flavin is labeled. Ligands are highlighted in *black sticks*, and the FAD cofactors are highlighted in *yellow*. Oxygen atoms are in *red*, and nitrogens are *blue*.

oxidizes 1 μmol of L-galactono-1,4-lactone/min, which is equivalent to the reduction of 2 μmol of cytochrome *c* (25). The reaction with molecular oxygen was assayed with a polarographic oxygen uptake assay using a Clark electrode in aerated buffer (0.25 mM oxygen at 25 °C) in the absence of cytochrome *c*. To identify the product of oxygen reduction, catalytic amounts of catalase (10 μg) or superoxide dismutase (10 μg) were added to the oxygen uptake assay mixture.

Stopped-flow Kinetics—The reoxidation of wild-type GALDH and A113G with molecular oxygen was studied by stopped-flow kinetics using an Applied Photophysics SX17MV instrument. All experiments were performed in 50 mM sodium phosphate, pH 7.4, at 25 °C. Solutions were made anaerobic by flushing with oxygen-free nitrogen. To scavenge final traces of oxygen, 1 mM vanillyl-alcohol and catalytic amounts of eugenol oxidase were added to the reaction mixture. Reduced enzyme was prepared by using 1.2 eq of substrate. The oxidative half-reaction was followed at 450 nm by mixing the substrate-reduced enzyme with buffer containing various concentrations of oxygen (0.25–1.25 mM). Obtained spectra were analyzed with the Pro-K software (Applied Photophysics), and single-wavelength traces were fitted to a double exponential function,

$$A_t = A_1 \times e^{(-k1 \times t)} + A_2 \times e^{(-k2 \times t)} + C, \qquad \text{(Eq. 2)}$$

where A is absorbance difference at 450 nm, k is the rate constant (s^{-1}), and C the absorbance off-set at 450 nm.

RESULTS

Structural Correlations with Oxidase Activity in the VAO Family—Most members of the VAO flavoprotein family are oxidases containing a covalently bound FAD cofactor (2, 4). Inspection of the structure-based multiple sequence alignment of the FAD domain of many VAO members yielded another striking correlation with oxidase activity. All known oxidases, with the exception of alditol oxidase and xylitol oxidase, contain either a glycine or a proline residue at a structurally conserved position near the C4a locus of the flavin isoalloxazine ring that is potentially reactive toward molecular oxygen. GALDH and other dehydrogenases, like cytokinin dehydrogenases and D-lactate dehydrogenase, contain an alanine, threonine, or isoleucine at this position. The occurrence of amino acid residues at the structurally conserved position near flavin C4a in both oxidases and dehydrogenases of the VAO family is shown in the supplemental materials.

Inspection of the known crystal structures of VAO members revealed that the residues at the conserved position near flavin C4a are located on the *re*-side of the flavin, opposite of the substrate binding site, which is on the flavin *si*-side in VAO members. In Fig. 1, the active sites of AldO and CO, the closest homologs of GALDH with a known three-dimensional structure are depicted. A high degree of conservation is observed between GALDH, AldO, and CO in the substrate binding site and the area around the isoalloxazine ring of the flavin. AldO, although an oxidase, contains an alanine (Ala-105), analogous to Ala-113 of GALDH, near flavin C4a, whereas CO contains a proline (Pro-186) at this position. To address the potential oxidase activity of GALDH, an A113G variant was constructed by site-directed mutagenesis to create more space near flavin C4a.

Spectral Properties—Mutation of Ala-113 in GALDH into Gly yielded a stable holoprotein, which could be expressed and purified in similar quantities as wild-type GALDH (7). During the purification procedure, a significant fraction of the mutant protein (about one-third) lost its FAD cofactor, but the apoprotein was readily reconstituted to the holo form with commercial FAD. The Ala-113 to Gly replacement gives small spectral perturbations in the flavin optical spectrum, the maxima at 376 nm and 450 nm are red and blue shifted, respectively, and the shoulder at 475 nm is less pronounced (Fig. 2). The molar absorption coefficient of the mutant protein is 13.0 $mM^{-1}\,cm^{-1}$ at 449 nm when compared with 12.9 $mM^{-1}\,cm^{-1}$ at 450 nm for the wild-type protein.

Catalytic Properties—The steady-state kinetic parameters of GALDH A113G for L-galactono-1,4-lactone were determined using both oxygen and cytochrome *c* as electron acceptor. The A113G mutation yields a striking increase in turnover rate with molecular oxygen, $k_{cat} = 42\ s^{-1}$ when compared with $0.17\ s^{-1}$ for wild-type, whereas retaining the cytochrome *c* reductase activity, $k_{cat} = 116\ s^{-1}$ *versus* $134\ s^{-1}$ for the wild-type protein (Fig. 3*A*). The Michaelis constant for L-galactono-1,4-lactone is slightly higher for the mutant protein than for the wild-type enzyme (Table 1). The steady-state kinetic measurements with oxygen and cytochrome *c* suggest that in the A113G variant, the reoxidation with oxygen is the rate-limiting step during catalysis, and not flavin reduction or product release as is typical in flavoprotein oxidases (26).

To determine whether hydrogen peroxide or superoxide is produced as a result of oxygen reduction by GALDH A113G, the oxygen uptake assay was performed in the presence of cat-

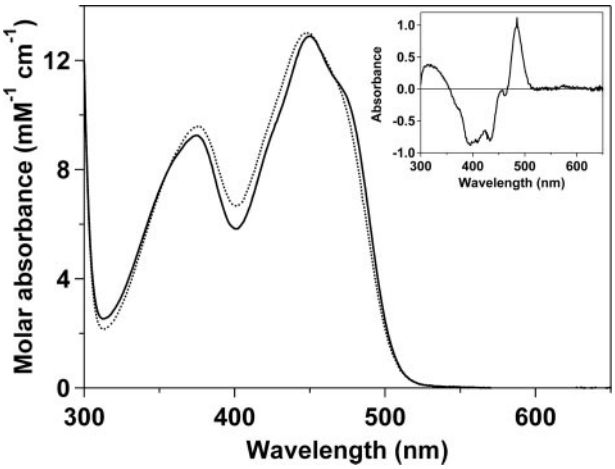

FIGURE 2. **Optical spectra of oxidized wild-type GALDH (*solid line*) and A113G (*dotted line*).** Spectra are taken in 50 mM sodium phosphate, pH 7.4. The *inset* shows the difference spectrum of wild-type GALDH and A113G.

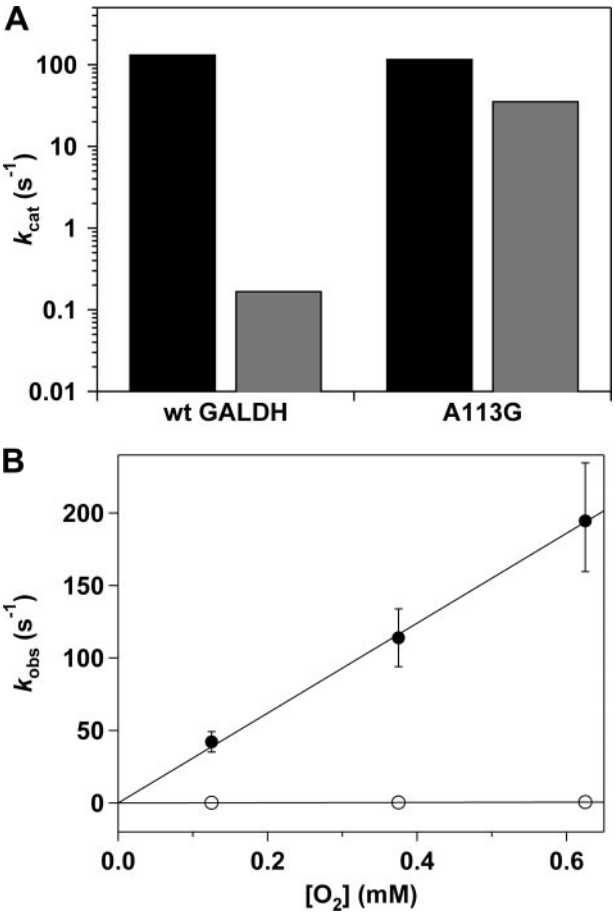

FIGURE 3. **Catalytic properties and oxygen reactivity of wild-type GALDH and A113G.** *A*, cytochrome *c* reductase activity (*black bars*) *versus* oxidase activity (*gray bars*) of wild-type GALDH and A113G. *B*, reoxidation of wild-type (*wt*) GALDH (*open circles*) and A113G (*closed circles*) by molecular oxygen. Substrate-reduced enzyme was mixed with 50 mM sodium phosphate, pH 7.4, containing various amounts of oxygen at 25 °C in the stopped-flow apparatus. Kinetic traces were fit to a double exponential function. The average rates ± the S.D. of at least seven traces are shown.

alase, which converts hydrogen peroxide into water and oxygen, or superoxide dismutase, which converts superoxide into hydrogen peroxide and oxygen. In the presence of catalase, the

TABLE 1

Kinetic parameters of wild-type GALDH and A113G for L-galactono-1,4-lactone using cytochrome *c* or molecular oxygen (air saturated buffer) as electron acceptor

	Wild-type[a]	A113G
Cytochrome *c* (50 μM)		
k_{cat} (s^{-1})	134 ± 5	116 ± 5
$K_{m,\,GAL}$ (mM)	0.17 ± 0.01	0.45 ± 0.03
$k_{cat}/K_{m,\,GAL}$ (mM^{-1} s^{-1})	7.7×10^2	2.6×10^2
Oxygen (0.26 mM)		
k_{cat} (s^{-1})	0.17 ± 0.02	42 ± 6
$K_{m,\,GAL}$ (mM)	ND[b]	0.46 ± 0.10
$k_{cat}/K_{m,\,GAL}$ (mM^{-1} s^{-1})	ND[b]	9.1×10^1

[a] Data from Ref. 7.
[b] ND, not detectable due to low oxygen consumption rates.

oxygen consumption rate was about half of the rate observed in the absence of catalase. Moreover, when catalase was added at the end of the reaction, about 50% of the consumed oxygen was regenerated. The addition of superoxide dismutase to the assay mixture had no effect on the oxygen consumption rate, confirming that hydrogen peroxide is indeed formed as a result of flavin reoxidation by the A113G variant. Also, for wild-type GALDH, no superoxide formation could be detected.

Oxidative Half-reaction—The kinetics of reoxidation of wild-type GALDH and the A113G variant by molecular oxygen were studied in more detail by mixing substrate-reduced enzyme with aerated buffer in the stopped-flow apparatus. Diode-array detection revealed that flavin reoxidation did not involve spectral intermediates in both variants. Deconvolution of the spectra taken during reoxidation of wild-type GALDH revealed a single phase, whereas A113G showed two phases during the reoxidation, a fast phase ($k = \sim 30$ s^{-1}) corresponding to flavin reoxidation and a second slower phase ($k = \sim 7$ s^{-1}) (data shown in the supplemental materials).

The effect of oxygen concentration on the reoxidation rate of A113G was studied by single wavelength detection. Traces were best fit to a double exponential function. The first fast phase represents the reoxidation event and shows a linear dependence on the oxygen concentration, giving a bimolecular rate constant of 3.4×10^5 M^{-1} s^{-1} for flavin reoxidation (Fig. 3*B*), a 400-fold increase when compared with wild-type GALDH, which has a bimolecular rate constant of 9.1×10^2 M^{-1} s^{-1}. Reoxidation in the presence of excess product only had a minor effect on the rate of reoxidation of A113G, suggesting that the data presented reflect the oxygen reactivities of the free reduced enzymes.

The second slower phase observed during the reoxidation of A113G accounts for maximally 10–15% of the total absorbance change. The corresponding rates were not dependent on the oxygen concentration and were around 5–10 s^{-1} in all cases. The nature of this phase is unclear; as it is slower than the observed k_{cat}, it might represent a non-catalytic event. Alternatively, the A113G protein preparation might consist of two populations, a fast reacting species and a slow reacting one, because the spectral difference in both processes is the same. A similar slow phase (around 5 s^{-1}) that is not dependent on the oxygen concentration was detected during the reoxidation of type II CO (17).

Reduction Potential—The protein structure around the isoalloxazine moiety of the flavin controls both the catalytic

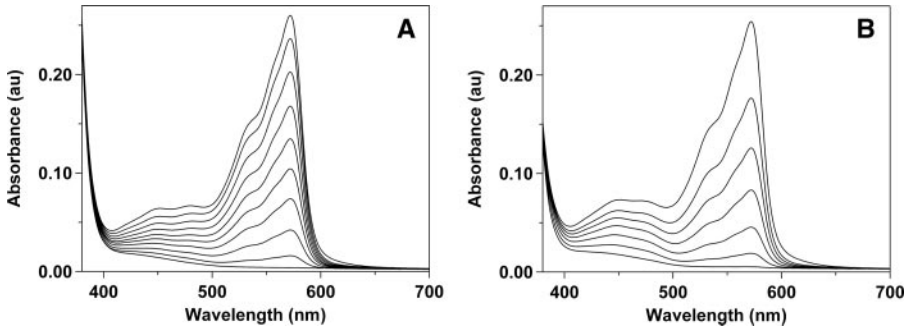

FIGURE 4. **Anaerobic reduction of 5 μM wild-type GALDH (*A*) and A113G (*B*) by the xanthine/xanthine oxidase method (23) in the presence of 5 μM resazurin in 50 mM sodium phosphate, pH 7.4, at 25 °C.** Spectra were taken at regular time intervals until complete reduction was achieved after 50 min. For clarity only selected spectra are shown. *au*, absorbance units.

function and the redox properties of the enzyme. To find out what is the basis for the observed increase in oxidase activity in the A113G mutant, the reduction potentials of wild-type GALDH and A113G were estimated using the xanthine/xanthine oxidase method (23), using resazurin/resorufin (E_m resorufin = −51 mV) as the reference dye. Both wild-type and A113G were reduced in a single two-electron step, without the formation of a semiquinone species (Fig. 4). The reduction via a single two-electron process fits well with the catalytic function of GALDH, the two-electron oxidation of L-galactono-1,4-lactone. The midpoint reduction potentials of wild type and A113G could be estimated from the log (E_{ox}/E_{red}) *versus* log (dye_{ox}/dye_{red}) plots and were −44 mV for wild-type GALDH and −63 mV for A113G. These values are in the same range as has been reported for other non-covalent flavoprotein dehydrogenases and oxidases (27), whereas covalent flavoproteins generally have higher reduction potentials (28, 29). The Ala-113 to Gly mutation lowers the midpoint reduction potential of GALDH by about 20 mV. Such a small decrease in reduction potential cannot account for the observed increase in oxidase activity.

DISCUSSION

GALDH from *A. thaliana* is one of the few members of the VAO flavoprotein family that reacts poorly with molecular oxygen (7). By performing a structure-based multiple sequence alignment (21) of the FAD domain of known VAO family members, we found a striking correlation between the presence of a Gly or Pro residue at a structurally conserved position near the C4a locus of the flavin and the oxidase activity of these enzymes. Replacing the corresponding residue in GALDH (Ala-113) with Gly increased the oxygen reactivity of GALDH with several orders of magnitude, yielding a catalytically competent oxidase.

All oxidases of the VAO family contain a covalently bound FAD (4). The properties of the GALDH A113G variant clearly show, however, that this feature is not essential for VAO members to acquire oxidase activity. Covalent flavinylation does not enhance the reactivity of the flavin with molecular oxygen but merely increases the flavin reduction potential, *i.e.* the power for substrate oxidation. The VAO H422A variant, which contains non-covalently bound FAD, has a much lower reduction potential than the wild-type enzyme but a nearly identical reaction rate with molecular oxygen (28). Similarly, cytokinin dehydrogenase contains covalently linked FAD, accordingly has a relatively high reduction potential (+8 mV), but reacts poorly with molecular oxygen (30). In agreement with the above considerations, we found that the A113G mutation in GALDH results in a minor lowering of reduction potential of about 20 mV but a 400-fold increase in oxygen reactivity.

Properly positioned positive charges are thought to enhance the oxygen reactivity of flavoenzymes, possibly by stabilization of the superoxide-flavin semiquinone radical pair generated after the first electron transfer from the reduced flavin to oxygen (12). For glucose oxidase, it was demonstrated that a protonated His (His-516) in front of the N5-C4a flavin locus is solely responsible for the observed fast reaction with molecular oxygen (k_{ox} = 1.5×10^6 M^{-1} s^{-1}) (31). Similarly, Lys-265 was found to be responsible for oxygen activation in monomeric sarcosine oxidase. Replacing this Lys with a neutral residue resulted in an 8000-fold decrease in oxygen reactivity (18). GALDH contains two basic residues (Arg-388 and Lys-456) at the *si*-face of the flavin ring that are possibly important for the reaction with oxygen. These residues are conserved in AldO and CO (Fig. 1), where they are involved in substrate oxidation (14). In monomeric sarcosine oxidase, the sites for substrate oxidation and oxygen reduction are also found on opposite faces of the flavin (32). As noted by Jorns and co-workers (18), such an arrangement might avoid steric crowding in the ternary complex.

The chemical reaction of flavoprotein oxidases with molecular oxygen is not completely understood but involves the transfer of one electron from the reduced flavin to oxygen, resulting in the formation of a superoxide anion/flavin semiquinone radical pair. Next, the radical pair can dissociate to release the oxygen radical, it can undergo a second electron transfer to produce hydrogen peroxide, or it can form a covalent C4a-(hydro)peroxyflavin species that can further dissociate to form hydrogen peroxide. This oxygen-flavin adduct is generally unstable but is a common feature in flavoprotein monooxygenases that use this intermediate to insert an oxygen atom into their substrates (33). For oxidases, it is not clear whether the C4a-(hydro)peroxyflavin is formed during the catalytic cycle. Recently, for the first time, such an intermediate has been experimentally detected in a flavoprotein oxidase: pyranose 2-oxidase (34). The oxygenated flavin species decayed to form oxidized flavin and hydrogen peroxide, consistent with the inability of pyranose 2-oxidase to perform oxygenation reactions.

Most flavoprotein oxidases show a linear dependence on oxygen concentration, reflecting a second order process, suggesting that the reaction is the result of a collision of oxygen and the reduced flavin cofactor and that oxygen can freely diffuse into the active site (12). For AldO, where no C4a-(hydro)peroxyflavin intermediate is detected during flavin reoxidation (35), it was argued that the methyl side chain of Ala-105, homol-

ogous to Ala-113 in GALDH, could hamper the formation of the peroxy-flavin intermediate by steric hindrance (14). AldO and GALDH show 25% sequence identity, but a high degree of conservation between these enzymes is observed in the area around the isoalloxazine ring of the flavin, including several residues in the substrate binding pocket. This supports that the A113G mutation in GALDH creates space for molecular oxygen to reach and react with the N5-C4a locus of the reduced FAD cofactor. The created space likely results in a cavity big enough to accommodate a molecule of dioxygen.

The acyl-CoA oxidases and dehydrogenases form another group of structurally similar flavoenzymes that use different electron acceptors. A distinct oxygen binding site near the C4a-N5 locus of the flavin was proposed for the acyl-CoA oxidases and assigned to the presence of a Gly (36, 37). The role of this residue in the oxygen reactivity was, however, never experimentally evaluated. Members of the FMN-dependent L-2-hydroxy acid oxidase family contain either a Gly or an Ala at the end of a four-residue loop in close proximity of the C4a-N5 locus of the flavin (12). Changing this Gly to Ala in L-lactate monooxygenase significantly reduced the oxygen reactivity (38), whereas for mandelate dehydrogenase, a slight increase was found (39). Based on these results, it was argued that the oxidase activity in this family might be controlled by steric effects through the relative positions of the FMN and the four-residue peptide loop. Members with a poor oxygen reactivity show a more constrained geometry around the N5 position of the flavin (39).

In conclusion, we have shown for the first time that it is possible to convert a flavoprotein dehydrogenase into a catalytically competent oxidase. Our findings are of general relevance for the design of suitable biocatalysts because oxidases do not require expensive co-substrates or regeneration systems (40). Removal of the Ala-113 side chain of GALDH creates space and allows oxygen to reach the reduced flavin. The presence of such a gatekeeper residue in dehydrogenases is a key factor for preventing oxidase activity in the VAO flavoprotein family. The question remains whether oxygen reaches the active site through defined oxygen tunnels or via multiple entrances as the result of protein breathing. What seems to be of key importance is the presence of space for molecular oxygen to reach and attack the reduced flavin.

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